An Address
ON
THE METHOD OF TISSUE CULTURE AND ITS BEARING ON PATHOLOGICAL PROBLEMS.*

(With Special Plate.)

BY
ALEXIS CARREL, M.D.
(From the Laboratories of the Rockefeller Institute for Medical Research, New York.)

THE attempts made during past years to apply the method of tissue culture to pathological studies did not meet generally with great success. This must not be attributed to the method itself, but merely to the fact that the techniques were not adapted to the requirements of physiological and pathological investigations. The early procedure, which was derived from the experiments of Ross Harrison and is still widely used, consisted merely in placing a fragment of embryonic or adult tissue in a drop of lymph, plasma, serum, saline solution, or other media, hanging in a sealed hollow slide. While surviving under these conditions, the cells are subjected to complex and obscure influences such as those of necrotic cells of their own type, living and dead cells of other types, and a medium which deteriorates spontaneously within a short time. Obviously, the factors responsible for the morphological or dynamic changes observed in the tissues cannot be analysed accurately. It is as impossible to study a problem of cell physiology with a fragment of fresh tissue in a hanging drop of plasma, serum, or other fluids, as it is to investigate the properties of bacteria cultivated in a medium of spontaneously varying composition and contaminated by other bacteria. It could hardly be expected that such imperfect procedure should lead to important results. Before a study of complex problems could be properly undertaken more elaborate methods had to be developed by which the interactions of pure strains of cells and the humours could be elucidated and expressed quantitatively, whenever feasible.

"When you can measure what you are speaking about and express it in numbers," wrote Lord Kelvin, "you know something about it, and when you cannot measure it, when you cannot express it in numbers, your knowledge is of a meagre and unsatisfactory kind. It may be the beginning of knowledge, but you have scarcely in your thought advanced to the stage of a science."

These words by one of the greatest of modern physicists apply as well to pathology as to physics, chemistry, or physiology. Biological phenomena must be reduced to their simplest terms, in order that their fundamental principles may be unveiled, and qualitative conceptions be replaced as far as possible by quantitative relations. Therefore I shall confine myself to speaking of the methods permitting the measurement of the interactions of the tissues and their medium, and, wherever practicable, the description of experiments made by the early procedure with impure cultures of tissues will be omitted, because these experiments have often led to erroneous conclusions. In this paper I shall briefly review the new techniques developed during the last few years, and some of the results already obtained.

Pure Strains of Cells.

The isolation and maintenance of pure strains of various types of tissues was the first and most indispensable step in the adaptation of the method of tissue culture to physiological research. The first pure culture of fibroblasts originated over twelve years ago from a fragment of the heart of a chick embryo, and was cultivated in embryonic tissue juice, which was discovered at about the same time to have the property of giving to fibroblasts an unlimited proliferating capacity. Pure strains of fibroblasts can be obtained easily from practically every tissue of fowls, dogs, etc., as connective tissue cells multiply more rapidly than the other cells, which spontaneously disappear within a few weeks. The fibroblasts grow as a dense tissue which doubles its volume in about forty-eight hours. The cells are polygonal or spindle-shaped, with long, sharp processes, and with an oval nucleus containing generally two nucleoli (Fig. 1). The strain, now over twelve years old, has produced a very large number of cultures and has been used in many experiments as a physiological reagent for detecting in serum and other fluids the presence of substances promoting or inhibiting cell multiplication.

Strains of lymphocytes and large mononuclears were obtained from cultures of white blood corpuscles. The polymorphonuclear leucocytes, endowed with greater activity, occupy the peripheral area of the culture. The inner zone is covered chiefly by lymphocytes and monocytes. After a few days the polymorphonuclear leucocytes progressively disappear. They are generally taken up by the lymphocytes and digested. Finally, the mononuclear cells alone remain (Fig. 2), multiply in the medium, and wander through it isolated or in columns, but never aggregate in a tissue. When they accumulate in large numbers, or are packed together by contraction of the medium, they generally die or become transformed into fibroblasts. The growth of the cultures is slow and the cells are less resistant than epithelial and connective tissue cells. No strain has lived for more than three months. But, as cultures of lymphocytes and macrophages free from polymorphonuclears are obtained from blood cells in a very short time, it is unnecessary to maintain the same strain indefinitely.

A strain of cartilage cells has been isolated from the pars cartilago sclerae of the eye of chick embryos by Fischer, and cultivated for more than three months. The hyaline substance disappeared and the small lymphocyte-like cells became transformed into large spindle-shaped cells, which formed thin membranes. The rate of growth was slower than that of fibroblasts and epithelium.

The isolation of a strain of epithelial cells was attempted many times without success. As the epithelium was always contaminated by a few fibroblasts which grew at greater speed, pure cultures could not be obtained. Finally, in this laboratory Fischer succeeded in growing a strain of epithelium from the few cells of the iris which adhere to the lens of a chick embryo. When multiplying on the surface of a plasma clot in a thin layer of embryonic juice, the cells assume the appearance of a continuous membrane (Fig. 3). Their rate of growth is slower than that of fibroblasts, and the cultures double in size in about three days. A strain of epithelial cells isolated by Ebeling almost two years ago has lost none of its characteristics. It does not dedifferentiate. The cells grow as a thin mosaic at the surface of the medium and manufacture a large amount of black pigment, similar to that of the cells of the iris. Ebeling and Fischer have observed them forming finger-shaped processes, like those found long ago by Burrows and myself in cultures of kidney and of thyroid gland more or less contaminated with fibroblasts, and those recently described by Drew. Fischer also showed that keratinization of the epithelial cells, attributed by Drew to the presence of fibroblasts, takes place in pure cultures. When epithelium is allowed to grow in contact with pure cultures of fibroblasts, it gives rise to gland-like structures, as has been shown by Ebeling and Fischer.

For several years attempts to isolate strains of thyroid, Malpighian, renal, and other epithelial cells remained unsuccessful. Our technique consisted in extirpating by section with a cataract knife small islands of epithelial cells, growing from embryonic tissues, and, in some cases, groups of cells uncontaminated by fibroblasts could be transplanted into a fresh medium. In order to obtain pure cultures of renal epithelium, Drew used an ingenious technique, which consists in protecting an island of epithelium with a drop of mercury and in killing the other cells by the rays of a quartz mercury lamp. The main objection to this procedure is that dead cells are left in contact with living cells, and that necrotic tissues may set free substances which modify the living structures more or less profoundly.

Important progress in the cultivation of epithelium has been made recently, when Ebeling isolated a pure strain of thyroid cells from a chick embryo and maintained it in active condition. Thyroid cells from a five months old strain grow equally well at the surface of the medium (Fig. 4) or in its depth. They easily digest the fibrin of the coagulum, but they multiply at a more rapid rate and are more resistant than pavement epithelium. Sometimes

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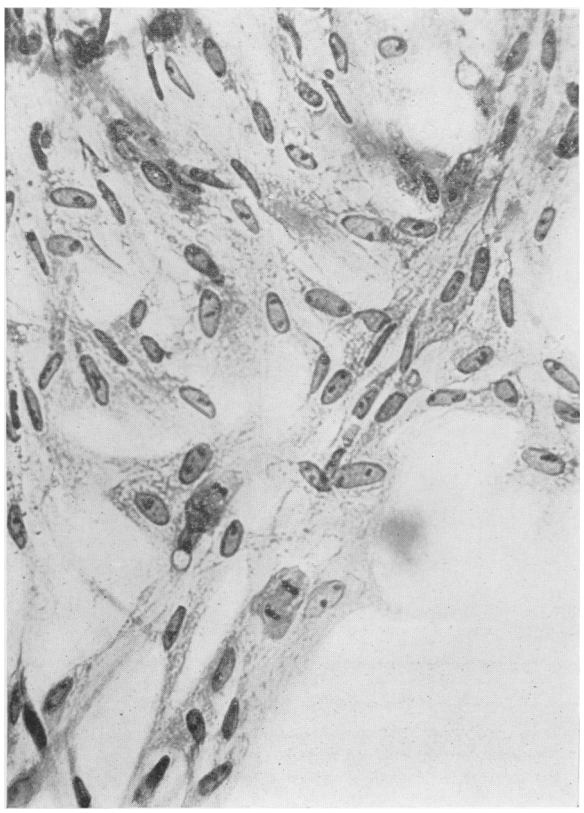

FIG. 1.—Pure culture of fibroblasts from a 12 years old strain.

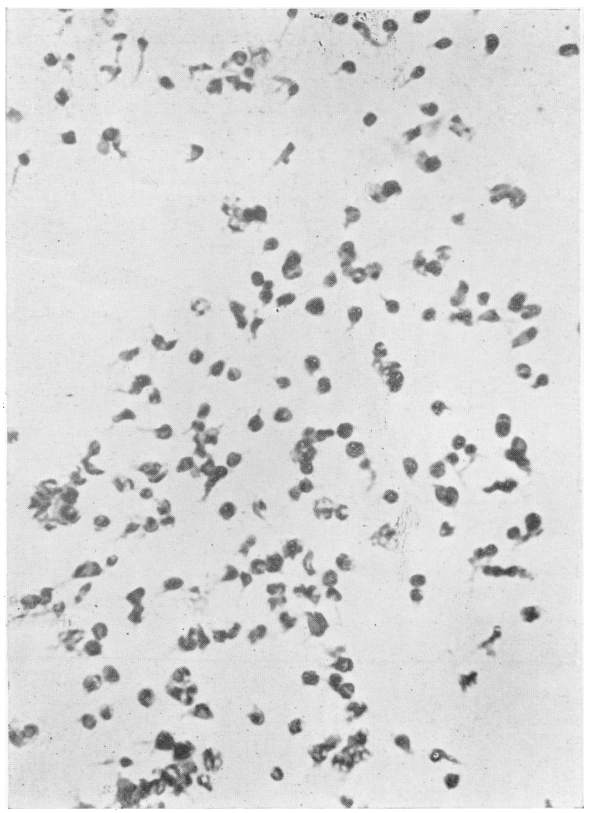

FIG. 2.—Pure culture of large mononuclear cells, twelfth passage.

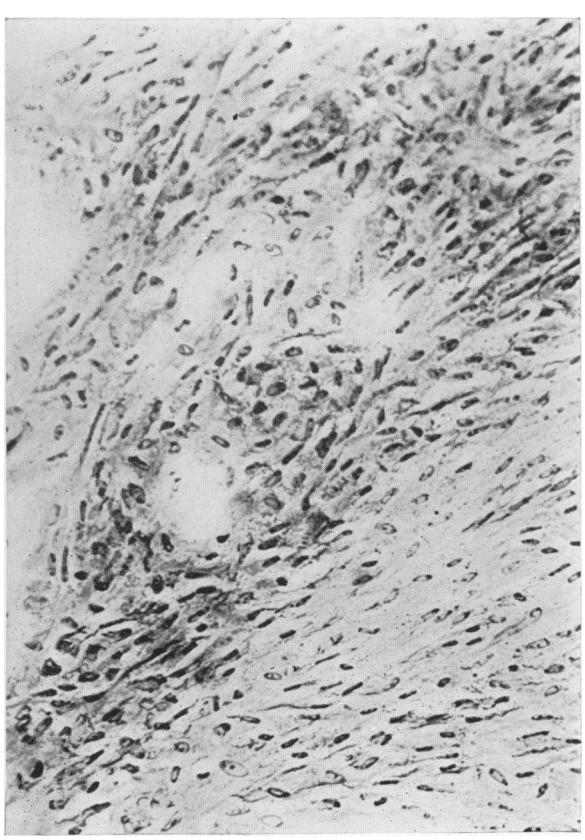

FIG. 3.—Pure culture of pavement epithelium from a 19 months old strain.

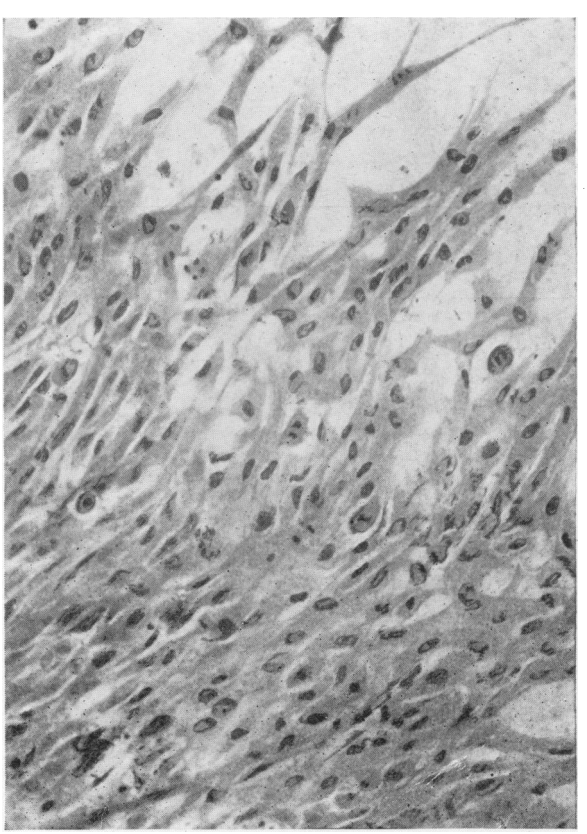

FIG. 4.—Pure culture of thyroid epithelium from a 6 months old strain.

they form acini with colloidal material in the centre. Generally they are more or less irregularly spread in the coagulum, and some drops of colloid substances with their characteristic appearance can be seen within the cells or near them in the medium. When pure cultures of fibroblasts are added to thyroid tissue the fibroblasts soon surround the thyroid epithelium, which organizes in a small gland showing acini filled with colloid substance. The inclusion of a fragment of thyroid strain in a pure culture of fibroblasts accelerates the proliferation of the fibroblasts during a number of passages. It is obvious that thyroid cells *in vitro* have the property of secreting substances which stimulate the growth of fibroblasts. Possibly these substances are identical with those contained in thyroid extracts, whose action on connective tissue I observed long ago, *in vivo* as well as *in vitro*.

The behaviour of this strain of thyroid cells and the persistence of their characteristics in spite of the absence of connective tissue have an important bearing on the theories of Champy and Drew regarding dedifferentiation of tissues *in vitro*. Long ago Champy observed that epithelial cells which migrate from fragments of kidney surviving in a drop of plasma lose most of their characteristics and return to an indifferent stage where epithelial and connective tissue cells cannot be distinguished from one another. He found also that the specific secretion of the guinea-pig prostatic cells soon disappeared when they were cultivated in serum. When connective tissue was present dedifferentiation of epithelial cells did not occur. He concluded that epithelium cultivated *in vitro* is bound to dedifferentiate when no connective tissue is present. More recently the same theory was defended by Drew. It was hardly possible to infer from those experiments, which were made according to the early technique and with impure cultures, that cells cultivated *in vitro* necessarily dedifferentiate. In Champy's experiments the tissues were not even in a condition of cultivation, but merely of survival, and the duration of the survival was very short. Dedifferentiation of epithelial cells in tissue cultures is far from being a necessary consequence of life *in vitro*. Since it is proven that pure strains of pavement epithelium and of thyroid epithelium maintain their essential characteristics after a long period of cultivation, the theory of Champy and Drew must be definitely abandoned.

Pure Strains of Cells from Sarcomas.

Some years ago several attempts were made to obtain pure cultures of malignant cells from Rous sarcoma, but no strains could be kept permanently. Losee and Ebeling tried also to obtain a pure strain of human sarcoma. After a few days the cultures were composed of apparently normal fibroblasts which ultimately died. Fischer recently published a procedure by which tumour cells cultivated with small fragments of muscle proliferated *in vitro* and kept their malignant characteristics. Cultures from a six months' old strain grafted into chickens brought about the growth of malignant tumours. During the past year I obtained pure cultures of cells from malignant tumours of different types—Rous sarcoma, spindle-cell sarcoma from the thoracic wall, round-cell sarcoma from the eye, and ovarian sarcoma.

As fibroblasts and amoeboid cells possess different cultural properties, pure cultures of both types of cells could be isolated from Rous sarcoma. The strains of fibroblasts were kept for more than five months in order to ascertain whether their malignant nature would persist. Cultures were inoculated into fowls at different intervals. The malignant characteristics generally disappeared after from two to four passages—that is, from four to seven days. A strain remained slightly malignant for several months in only one instance.

Wandering cells in pure cultures had the appearance of monocytes. They spread slowly into the medium, always remained isolated during their migration, and never formed a tissue. The inoculation of these cells into fowls gave rise to tumours which had the characteristics of the original tumour. An eighteen-day-old culture composed exclusively of amoeboid cells produced a rapidly growing sarcoma in a short time. So far it has not been possible to isolate polymorphonuclear cells from sarcomas and test their power of producing tumours.

Preservative and Nutritive Media.

By the study of cells in pure cultures it was determined whether a given medium is nutrient or only preservative. In the early experiments the tissues were supposed to live on the constituents of the lymph or plasma used as a culture medium. But soon Lewis found that proliferation of embryonic tissues takes place as readily in saline solution as in serum, and that the tissue fragments cultivated in plasma do not increase in mass. Although the minute fragment of embryonic heart from which originated the first pure strain of fibroblasts proliferated for more than three months in plasma, it progressively decreased in volume. But as soon as embryonic juice was added to the medium the tissue began to grow. These and other experiments led to the conclusion that cells cultivated in plasma do not feed on the serum constituents but on substances contained in the tissue itself. Recently, the function of plasma and serum in the nutrition of fibroblasts was ascertained in extensive series of experiments made on pure cultures of fibroblasts with both techniques, repeated passages of the tissue in the medium, and changing the medium while the tissue grows undisturbed in a flask. These experiments rendered evident the fact that fibroblasts live no longer in Tyrode containing different dilutions of serum than in pure Tyrode solution. When the amount of serum is large the cells die sooner than in saline solution. If a small quantity of embryonic tissue juice is added to the medium the growth of fibroblasts and duration of their life is in no way influenced by the presence or absence of serum. Even if embryonic juice contains some growth-stimulating hormones these hormones fail to enable the fibroblasts to use the nitrogenous constituents of serum. But fresh embryonic heart can live in plasma much longer than a pure culture of fibroblasts because the disintegrating tissues, as Burrows has shown, set free substances which are used by fibroblasts for the synthesis of protoplasm. The proliferation of cells cultivated in plasma depends on some nitrogenous material stored within the cells and the tissues themselves. Serum proteins are not used by fibroblasts and epithelium as a source of nitrogen. Therefore, plasma and serum cannot be considered as a culture medium for those cells, in the proper sense of the word.

Several other fluids were supposed to be nutrient for tissues *in vitro*. Chicken bouillon is a part of the medium called Locke-Lewis solution, but it cannot be used by fibroblasts any more than serum. In some experiments made with the early technique the action on fragments of embryonic heart of a number of amino-acids was found to be toxic. But it was disclosed recently that amino-acids under the same concentration as in the blood have no poisonous effect on fibroblasts and epithelial cells in pure cultures, and that some of them increase the rate of cell migration and multiplication. But they are not used as a source of nitrogen by the tissues, which die as rapidly as in pure Tyrode solution. Egg-white allows a longer survival of the cells, and sometimes the fragments of tissue increase slightly in size. Crystallized egg albumen in a concentration similar to that of albumin in the serum does not allow an increase in mass of the connective tissue.

These experiments definitely proved that fibroblasts and epithelium do not synthesize protoplasm *in vitro* from nitrogenous constituents of plasma, serum, bouillon, or amino-acids, and that in these media they are in a condition of survival and not of cultivation.

Fibroblasts and epithelial cells may be considered as in a true state of cultivation only when embryonic tissue juices are present in the medium. Whenever embryonic juices are added to the medium of fibroblasts or epithelium the mass of the tissue begins to increase. As long as the cells are supplied with embryonic juice they synthesize new protoplasm and build up unlimited amounts of tissue.

This explains why the medium recently advocated by Drew is nutrient. According to my early experiments, and also some recent ones, it seemed that extracts of sarcoma, adult muscle and glands, leucocytes, and even of heterologous tissues, were endowed with a growth-promoting action similar to that of embryonic juices. In a recent article we reported the results of a study of the action of the juices from homologous and heterologous tissues on pure cultures of fibroblasts, not only for a few days, but for long periods of time. The effect of these juices, which may be practically similar for a few days, differs widely when their action is prolonged. Extracts of adult chicken organs, like embryonic tissue juice, at first determine an increase in the mass of the pure cultures of chicken fibroblasts, but, after a few passages, the fibroblasts cease multiplying and ultimately die. It is probable that adult tissues, as well as serum, contain substances which are toxic for the homologous cells, and progressively overcome the effect of the growth-promoting compounds. The activating effect attributed recently to adult gland and sarcomatous tissue by Drew entirely corroborates our early experiments. There is no marked difference between homologous and certain heterologous adult tissue extracts in their effect on fibroblasts, as demonstrated lately by Ebeling and myself. By contrast, heterologous embryonic

juices may act on chicken fibroblasts in the same manner as chick embryo juice. It appears that fibroblasts can synthesize protoplasm from substances contained in certain foreign embryonic juices.

Several years ago aqueous extracts of sarcoma were found by Burrows and myself to enhance cell multiplication, apparently in the same way as embryonic tissue juice does. This fact has recently been confirmed by Drew. But the action of extracts of sarcoma is more complex than was thought at first. When the cultures are kept under observation for several passages, the rate of cell multiplication, which is at first increased, becomes slower and the fibroblasts may ultimately die. Therefore the action of the juices from tumours can be compared only in a superficial way to that of embryonic tissue juices.

While fibroblasts and epithelial cells remain in a true condition of cultivation only in homologous and certain heterologous embryonic tissue juices, lymphocytes behave differently. In embryonic tissue juices they display great activity at first, but die after a short time. On the contrary, if the medium is composed of diluted serum, they remain alive and spread over a large area for more than a month. It is obvious that they build up protoplasm from some constituents of serum, while neither fibroblasts nor epithelial cells possess this property.

But it is not proved that such striking differences exist within the organism. Tissues within the organism may continuously receive substances which circulate in very high dilution in the blood, while *in vitro* these substances are soon exhausted and cannot manifest their action. However, there is some reason to believe that in the adult animal connective tissue does not find in plasma the substances necessary for its multiplication. If endothelial cells were capable of proliferating in blood plasma, they would soon fill the lumen of the vessels. Connective tissue is at rest in practically every part of the adult organism, probably because it does not find in interstitial lymph and plasma the necessary growth-promoting substances. Although fibroblasts do not proliferate spontaneously *in vivo*, they may multiply around foreign bodies or regenerating structures, when leucocytes secrete, or necrotic cells set free, substances similar to those which are found in tissue juices. These substances would be the substratum of the formative stimulus which is supposed to impart growth energy to regenerating tissues.

The nature of the nitrogenous compounds present in embryonic juice and used by epithelial and connective tissue cells in the building up of protoplasm is still unknown. But some of their properties have been studied during the last twelve years. Embryonic tissue juice loses its growth-promoting effect after it has been shaken for several hours, or heated at a temperature of 65° C. for a few minutes. The substances which promote cell multiplication are as sensitive to heat as alexin and sensitizer. They are partly destroyed at 56° C. in thirty minutes, and when left in an incubator at 38° C. for forty-eight hours their action is greatly weakened. They are unstable and easily destroyed by agents other than heat. They are sensitive to the action of radium and x rays. Spontaneous deterioration occurs also under the influence of still unknown factors. The decrease in the pH, which occurs spontaneously even at low temperature, prevents their stimulating action. But even when the pH does not vary, embryonic juices which have been kept in the refrigerator for one or two weeks lose some of the growth-promoting power. Filtration through a Berkefeld filter partly deprives the juices of their growth-activating substances, and these substances disappear completely after filtration through a Chamberland filter. These facts have recently been confirmed by Drew. Many attempts have been made to isolate from the embryonic tissue juices the substances used as a nitrogenous food by fibroblasts. Fractional precipitation by alcohol, acetone, and other substances did not lead to the separation of a more active moiety. The growth-promoting substances always remain with the protein precipitate. They may consist of some growth-stimulating hormones of the class called by Gley harmozones, and of other substances which supply fibroblasts and epithelium with the nitrogen required for multiplication, or only of the latter. These nitrogenous compounds, secreted by certain cells and used as food material by other tissues, are called trephones—from τρέφω, I feed.

It is obvious that the osmotic tension and the concentration in hydrogen ions of the medium must be similar to those of the humours of the organism. A convenient method was developed by Felton for measuring the hydrogen ion concentration of the medium. The optimum pH for the growth of pure cultures of fibroblasts was determined by Fischer. It was found to be between 7.4 and 7.8 for chicken fibroblasts. But the cells could live for four or five passages at a pH of 5.5 and for eight or nine passages at a pH of 8.5.

Methods for the Preparation of the Cultures.

It is well known that embryonic tissue may grow on the fibrin network of a plasma coagulum, on the surface of the cover-glass in a drop of lymph, serum, or saline solution, and on agar. But even in a fluid containing all the substances necessary for cell multiplication no growth occurs if the cells do not find the proper support on which to creep. The finest details of the cells spreading on a cover-glass in a saline solution may easily be observed, and this technique is excellent for cytological studies, as shown by Lewis. When the rate of growth of tissues must be accurately measured, and when the cultures must be transferred from medium to medium with as little disturbance of their structure as possible, the cells require a scaffold which offers sufficient resistance to handling with instruments.

Fibrin makes an excellent support for cells, which grow incomparably better and more regularly than on agar. The transfer of the tissues from medium to medium is possible without disturbing the cells, as they are embedded in and protected by the fibrin. The main objection to the use of fibrin is that in the early technique it could be obtained only from plasma, and the serum constituents could not be removed. Therefore it was impossible to observe the effect of simple saline or other solutions on the growth of cells. This disadvantage has been overcome by a method for obtaining and keeping fibrinogen suspension or solution, which is added to the fluid medium. At first the digestion of the fibrin prevented the use of serum-free media, but this obstacle was met by adding traces of sodium linoleate or egg-yolk to the fluid. A fibrin coagulum deprived of serum may also be obtained by washing a thin plasma clot in the bottom of a flat flask with Ringer or Tyrode solution.

A medium prepared according to the early technique deteriorates spontaneously within a short time. As the activity of the cells depends in a large measure on the composition of the pericellular fluid, no precise relation between a given factor and the changes in the morphology or the growth energy of a tissue can be established if the medium undergoes progressive and unknown modifications. The deterioration of the medium is due to the spontaneous destruction of thermolabile growth-promoting substances contained in tissue juices and serum, and to the accumulation of waste products from the tissues. This deterioration could be prevented by the cultivation of the cells in a continuous stream of oxygenated medium. But although Burrows succeeded in circulating serum at the surface of a plasma clot, no practical method has been evolved from those early experiments. The first technique by which cells could be kept indefinitely in a condition of constant activity consisted in removing the tissue fragment frequently from its medium, washing it in Ringer solution, and transferring it to a fresh medium. As the nutritive substances contained in the medium do not undergo marked disintegration in forty-eight hours, and as the accumulation of waste products has but a very slight action on the growth of the tissues during that period of time, the cultures, when transferred from medium to medium, live under practically unchanged conditions. This method has several disadvantages—for example, it is laborious and requires training to extirpate the tissues from the medium and transfer them to a new medium; only a small amount of tissue can be handled; and some tissues do not stand the passages well. While strains of fibroblasts and epithelial cells can be maintained indefinitely in an almost constant condition of activity, lymphocytes do not resist the frequent passages as well as other cells. Furthermore, cell secretions which require the cultivation of large amounts of tissue under constant conditions cannot be studied.

A second technique has been developed by which the culture medium may be renewed frequently without mechanical disturbance of the tissues or danger of bacterial contamination. Special containers have been made in the form of flat round flasks with oblique necks. They belong to different types, some being used for measuring the rate of growth of tissues, and the others when cytological examinations are required. The size of the flasks varies from 5 to 8 cm. in diameter, which makes possible the use of large amounts of tissues and medium. By means of spatulas the tissues may

be handled conveniently inside the flasks. The principle of the method is to use a medium composed of two parts, one solid and the other fluid, the latter being renewed as often as necessary. The solid medium is composed of coagulated plasma in which the tissues are embedded and which covers the bottom of the flask in a thin layer. The fluid medium, which bathes the solid medium continuously or not as desired, is composed of Tyrode solution in which are dissolved the substances whose action on the tissues is being investigated. The fluid medium diffuses its nutrient principles in the coagulum and also receives the katabolic products of the tissues. As it can be changed every day, the tissues grow on the bottom of the flask, without being disturbed, in a medium which varies but little in composition. With this technique, epithelial cells, fibroblasts, and leucocytes can be observed in a condition of uninterrupted growth for about a month, after which they must be transferred into a fresh medium.

Methods for Measuring the Rate of Growth of a Tissue and Detecting Growth-promoting or Inhibiting Substances in a Fluid.

As the action of a given substance on a tissue must be measured by its effect on the growth of a pure strain of cells, it was ascertained whether two parts of a tissue fragment may be caused to grow at identical rates in identical media.

A minute study was made of the technical conditions which are required for the uniform growth of pure cultures of fibroblasts. When the technique is carefully carried out the differences in the rate of growth of fibroblasts in identical media are slight. But the technique is delicate and, in untrained hands, the experimental errors are of such magnitude as to render the results worthless.

The measurement of the rate of growth is made in the following manner: The slides are placed in a projectoscope and the outlines of the tissues are drawn immediately after the preparation of the cultures, and after forty-eight hours' incubation (Fig. 5). The areas of the original fragment and of the new growth are measured with a planimeter and the ratio of the area of the new growth to that of the original fragment is calculated. This relative increase of

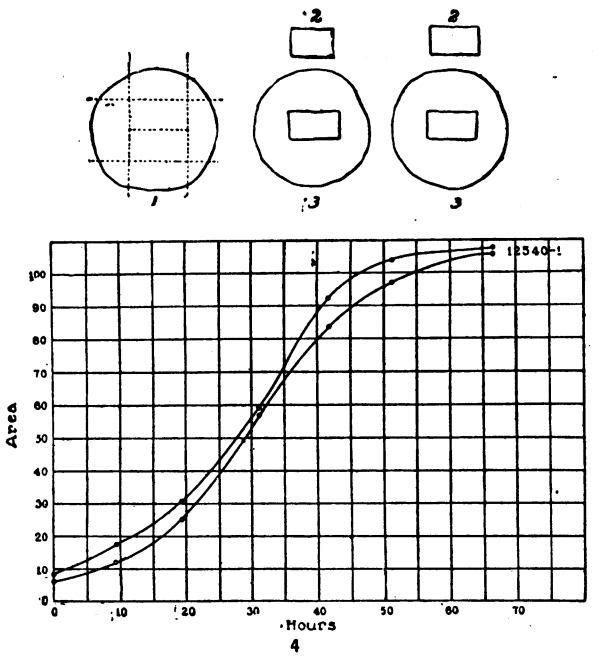

FIG. 5.—1, Division of a pure culture of fibroblasts into two fragments of equal size. 2, Tracing of the fragments in the projectoscope immediately after preparation of the culture. 3, Tracing after forty-eight hours, showing the area of growth. 4, Curve expressing the growth of two fragments of fibroblasts during sixty-seven hours.

pure cultures of fibroblasts and epithelium in forty-eight hours expresses the rate of growth. The rate of growth, the increase in mass of the tissues in a number of passages, and the duration of life of the cultures indicate whether the medium is nutrient, non-nutrient, or growth-stimulating without being nutrient. When the tissues are cultivated in large flasks, the increase is measured in the same way, and, as the growth is uninterrupted, it may be expressed graphically in a simple manner. The curve expressing the rate of growth of fibroblasts and epithelial cells in a nutrient medium is a parabola (Fig. 6). If the medium is non-

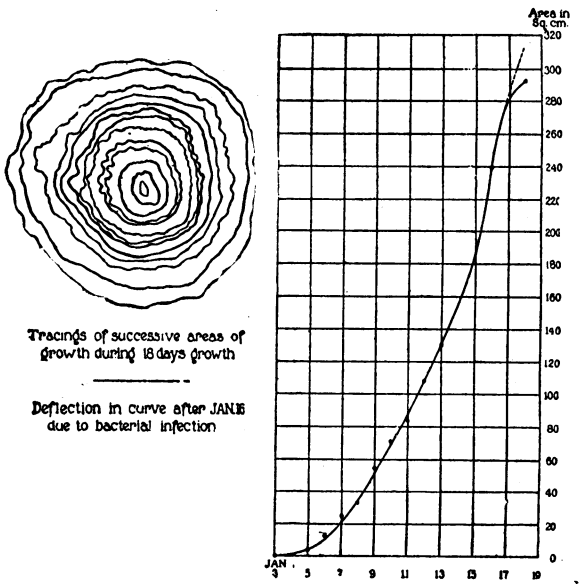

FIG. 6.—Tracings and curve showing the growth of a pure culture of pavement epithelium in a D-5 flask.

nutrient, the curve is S-shaped. Modifications in the form of this curve indicate whether the medium contains substances which activate or depress the rate of cell multiplication. The technique cannot be used for measuring the activity of lymphocytes, because they never grow as a tissue, but always remain at certain distances from one another and invade the culture medium on several planes. It is possible to use strains of epithelial and connective tissue cells as physiological reagents in order to detect in the humours the presence of substances promoting or depressing cell multiplication, and to express the action of these substances in terms of unit area of growth.

Methods for Ascertaining the Growth Energy of a Tissue.

The inherent growth energy of a given tissue can also be ascertained. It is obvious that the growth energy of a tissue *in vivo* may undergo wide variations under the influence of several factors, and chiefly of age. The curves of Minot demonstrated this long ago. The growth energy of the fibroblasts of a given organ is greater in an embryo than in a young animal, and fibroblasts in a nutrient medium are more active after twenty-four hours than after seventy-two hours. It is also known that a tissue may increase in growth energy by cultivation in a medium containing a large amount of embryonic substances. Therefore the inherent growth energy of a tissue at a given instant is a function of several independent variables: its growth energy at the preceding instant, and the concentration in the pericellular fluid of the substances which increase or decrease cell activity. It could be ascertained by the activity displayed during an instant by the cells in a medium containing neither activating nor retarding substances, if such a measurement were possible. But the

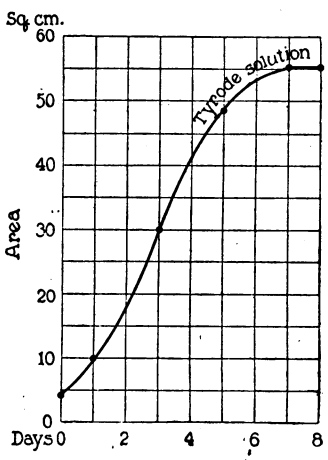

FIG. 7.—Residual activity of a pure culture of fibroblasts.

residual energy, which was found to vary at the same time as the inherent energy, may be used as a measurement of the latter. The residual energy (Fig. 7) is expressed by the duration of life and the proliferative activity of the cells in Tyrode solution.

Applications of the Methods to the Study of Physiological and Pathological Problems.

The method of tissue culture, although still in its infancy, is already a powerful instrument for the investigation of the relations of the tissues and the humours. It is capable of reducing certain phenomena to their simplest terms, since the action of a given substance on a given type of cells and the interactions of known types of cells can be studied for long periods of time. Thus it offers possibilities for the study of some problems which are beyond the reach of any other methods of research.

The behaviour of pure strains of tissues under various cultural conditions has shown that the rate of cell proliferation is not determined by the energy derived from the ovum, but is a function of the concentration in the pericellular fluid of substances which have the power of promoting or inhibiting cell proliferation. As long as the proper amount of food material is given to connective and epithelial tissues and the katabolic products are removed, cell proliferation goes on. Its velocity increases or decreases according to the composition of the medium, and remains constant in a medium of constant composition.

The strain of fibroblasts, which I obtained more than twelve years ago, is fully as active to-day as at the end of its first year. There is no change in the rate of growth of the strain of pavement epithelium isolated by Ebeling eighteen months ago. These tissues can be considered as potentially immortal. But the accumulation of katabolic products, the disappearance of the nutrient principles, the presence of toxic substances, etc., may bring about some morbid changes in the cells and eventually their death. It is probable that within the organism the pathological processes, which involve a resumption or decline of cell activity, are due to similar causes.

The methods used for measuring the amount of tissue produced under the influence of a given factor have permitted the detection and study of two classes of substances which probably play an important role in pathology. These substances that promote and restrain the growth energy of tissues are found in blood serum and in embryonic and other tissues.

Growth Depressing Action of Serum.

Serum not only fails to supply fibroblasts and epithelial cells with the substances necessary for multiplication, but it inhibits their proliferation and shortens their life *in vitro*. Although lymphocytes find in serum the substratum for multiplication, their activity is depressed by a large amount of this fluid in the medium.

The apparent acceleration of growth which occurs when plasma is diluted with a saline solution is caused merely by a decrease in the inhibiting action of serum under the influence of dilution. Although this phenomenon was studied long ago by Burrows and myself, by Lambert, and by Ebeling, its mechanism has remained unknown until the discovery of the growth-inhibiting action of serum on the multiplication of fibroblasts and epithelium.

As a rule, the restraining action of serum becomes more pronounced after serum has been shaken or heated. The agents which inactivate serum also render it more inhibiting for homologous fibroblasts. It seems that serum contains certain growth-promoting substances which, like alexin, sensitizer, and embryonic trephones, are destroyed by shaking and heating, and oppose the inhibiting effect on fibroblasts of other substances that resist heat and shaking. The study of the properties of a precipitate obtained by bubbling CO_2 through serum showed that serum contains two antagonistic substances, the growth-promoting ones always being weaker in their action than the growth-inhibiting.

The growth-restraining power of serum varies with the age of the animal. Serum never activates the multiplication of fibroblasts, even when obtained from a very young animal, but does not depress growth in the latter case. However, a short time after birth the serum becomes slightly inhibiting. The rate of cell multiplication and the duration of the life of a pure strain of fibroblasts in plasma decrease in function of the age of the animal from which the plasma is obtained, at first very rapidly and in later years very slowly (Fig. 8). The curve expressing this phenomenon resembles the growth curves of Minot, and that of the variations of the index of cicatrization in du Nouy's formula in function of the age of the patient. As no growth-activating power can be detected in the serum of very young animals, it appears that, during the whole life, the growth-inhibiting substances contained in plasma have a stronger effect on fibroblasts than the growth-activating ones, and that the effect of the latter is entirely shielded by that of the former. The increase of the inhibiting action of serum in the course of life is due to complex causes which were submitted to an experimental study. The conclusion of these experiments was that the increased inhibiting action of serum from old animals is due to the loss of growth-promoting substances and, at the same time, to the enhanced power of the growth-inhibiting factors. The growth-promoting substances probably come from gland secretions which decrease in old age. The enhanced growth-restraining effect may be due to the change in the properties or the quantity of the serum proteins. Hatai has shown that the concentration of proteins in rat serum increases with the progress of age. The same phenomenon occurs in fowls. But when the serum of an old fowl is diluted so that its protein concentration is practically equal to that of a young fowl, its growth-promoting power, which has decreased markedly, is still higher than that of the young animal. While increase in protein concentration is one of the mechanisms by which age acts on the inhibiting power of serum, other factors certainly play a part in the phenomenon.

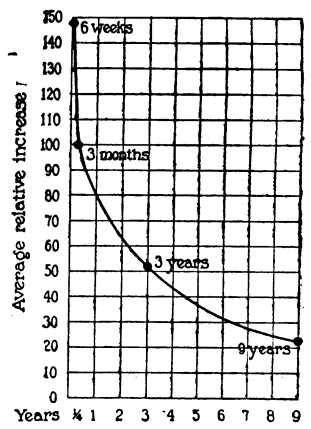

FIG. 8.—Curve showing the variations of the pure cultures of fibroblasts in serums from fowls of different ages.

The property of the blood serum of becoming more growth-inhibiting with advancing years was used as the basis of a technique for measuring age. The ratio of the rate of cell proliferation in serum to that in Tyrode solution is called growth index. It seems that it varies as an exponential function of the time. The value of the growth index decreases rapidly during the first months of life and slowly in old age. As chicken fibroblasts respond to the inhibiting power, not only of homologous, but also of certain heterologous serums, they may be used as a reagent for the detection of the modifications brought about by age in the blood of dogs.

When the growth index is adjusted to be 1.00 in dogs about two weeks old, it becomes rapidly lower, and variations from 1.00 to 0.70 and even 0.50 can be observed within two years. On the contrary, in old animals the variations of the growth index are small. In animals 8 or 9 years old its value varies generally between 0.2 and 0.1. In still older dogs the technique is not sensitive enough to show differences larger than these which may be due to experimental errors.

The growth index varies under the influence of conditions other than age. The serum becomes more inhibiting in the course of a general infection, after the spontaneous development of an abscess, or after an injection of turpentine, and returns to its normal condition when the animal recovers. The serums of animals which reach the cachectic stage of a malignant tumour may show an increase in their growth-inhibiting action. The variations of the growth index give an indication of the condition of the humours and of their possible action on the leucocytes and tissues. It is probable that some relations will be found between certain diseases and the growth-inhibiting properties of blood serum in adult and old organisms.

Embryonic and Leucocytic Trephones.

Embryonic tissue juice contains certain substances which give to fibroblasts and epithelial cells the power of proliferating indefinitely *in vitro*. This fact indicates that embryonic cells have the property of transforming some

material of the egg or the blood serum of the mother into growth-promoting substances. These substances are also found in extracts of leucocytes and of adult tissues such as thyroid, brain, kidney, etc. They are called trephones because they are used by certain cells as food material. They differ from the hormones that stimulate cells to activity without possessing any nutritive property. Both trephones and hormones may be required for the growth of tissues. Epithelial and connective tissue cells that do not obtain from blood serum the material necessary for the synthesis of protoplasm demand for that purpose the trephones contained in embryonic tissues, lymphocytes, and other cells. Leucocytic trephones undoubtedly have an important function in physiological and pathological processes. We found that extracts of leucocytes contain growth-activating substances, and that serum obtained from cultures of leucocytes promotes cell multiplication. Leucocytes secrete *in vitro* and *in vivo* substances that determine the multiplication of fibroblasts. Since lymphocytes are capable of multiplying in serum, they probably synthesize growth-activating substances from certain constituents of serum. They can be considered as mobile unicellular glands which bring to connective and epithelial cells the principles necessary for the synthesis of protoplasm.

This function of the lymphocytes was clearly understood by Renaut. More than thirty years ago he wrote that lymph cells bring to fixed cells the food material that they require, and that the life of the organism depends on their function. Jolly also believes that lymphocytes store important chemical substances which are used by the organism. The observations of Cramer, Drew, and Mottram have added a great deal to the evidence of the role of the lymphocytes in the nutrition of the body. It is shown by our experiments that lymphocytes retain through life the property of storing embryonic growth-promoting substances or trephones, which may cause a resumption of cell activity when needed.

The white cells of the blood are evidently endowed with a double function—defending the tissues against bacteria, foreign substances, and necrotic material, and promoting cell proliferation.

It is well known that pathological processes are often characterized by a resumption of activity of tissues that are more or less in a resting condition. In the healing of wounds and repair of fractured bones, and in the development of tumours, cells that have ceased multiplying, possibly for many years, recuperate their embryonic growth energy.

There has been much speculation concerning the mechanism that determines an increase of the inherent growth energy of a tissue when it is needed. But the explanation of this phenomenon was essentially incomplete, as the conditions responsible for cell multiplication were unknown. Although all the causes of tissue growth are far from being understood, we already know from observations of pure cultures of cells that growth energy depends on certain substances present in the pericellular fluid. Within the adult organism fibroblasts are not supplied with these substances. Since they do not obtain from serum the substances required for the synthesis of protoplasm they must receive them from another source. It is difficult to understand how an inflammatory irritant that, according to Virchow, would be responsible for the resumption of cell activity in pathological processes, could furnish the cells with the material needed for proliferation. The decrease in tissue tension, which Ribbert thought was the cause of the increased growth energy of a cicatrizing tissue, does not explain the production of new tissue. Weigert, while rejecting the hypothesis of Virchow, could not give any satisfactory explanation of the fact that injury of a group of cells is followed by proliferation of other cells.

Since cell growth energy depends on the concentration of growth-promoting substances in the pericellular fluid, it is probable that pathological tissue growth can be attributed to the presence of certain chemical substances, as foreseen by Welch many years ago. The substratum of formative stimulus may consist of trephones secreted by leucocytes or set free by dead muscle or gland cells and capable of supplying tissues with the food material required for cell multiplication.

This hypothesis satisfactorily explains the mechanism of wound healing and of several other phenomena. It is known that an aseptic wound begins to cicatrize after two or three days. But a wound completely free from debris of tissue and blood clots and absolutely protected from outside irritation does not heal. Thus, regenerative stimulus does not depend on a release of tissue tension. Neither does it come from tissue injury. It seems that Virchow was right in attributing cell growth to an inflammatory irritant. But when a wound, instead of being protected against external irritation, is covered with a slightly irritating dressing, such as turpentine, a few staphylococci, or dry gauze, cicatrization may start in less than two days. The action of the irritant on the cells is not direct. Gauze, turpentine, or bacteria have never been observed *in vitro* to produce an increase in the rate of multiplication of fibroblasts. They probably set in motion a mechanism which determines cell proliferation. The effect of every mild irritant is to bring about the invasion of the tissue by leucocytes which are known to have the power of promoting cell proliferation. We may assume that the resumption of cell activity in wound healing is due to the trephones set free by the leucocytes. Then Virchow's opinion was not entirely correct, since an inflammatory irritant initiates cicatrization not directly, but by the intermediary of the leucocytes.

Regeneration in lower organisms may also be determined by trephones. Jacques Loeb observed that, in Tubularia, endodermic cells gather at the end where a new polyp is about to form, and probably set free substances required by proliferating cells. The rapid growth of limbs in metamorphosing tadpoles is aided, according to Jordan and Speidel, by the presence of lymphocytes. Although it is certain that regeneration takes place through more than one mechanism, the trephones set free by leucocytes and other cells undoubtedly contribute to the resumption of cell activity within the adult organism.

When cells degenerate and die, as in myocarditis or interstitial nephritis, for example, they are replaced by fibrous tissue. Fibrosis is known to follow gland and muscle cell destruction. Since these cells set free substances that can be used as food material by the fibroblasts, the process of sclerosis appears to be automatic. The fibroblasts may synthesize new protoplasm from the principles diffusing from dying or dead cells and from leucocytes. The activation of fibroblasts, observed by Leo Loeb, under the influence of thyroid cells during regeneration of the gland *in vivo*, was probably due to the secretion of trephones. In many instances the resumption of cell activity that brings about adaptation in certain pathological processes can be satisfactorily explained by the presence of trephones in tissue fluids. The waves of cinesis that, in Dustin's experiments, follow the waves of pycnosis initiated by the injection of a foreign protein, are caused probably by substances set free by the dying cells.

The growth-restraining effect of serum and interstitial lymph, which does not allow fibroblasts to proliferate within the adult organism, must disappear when cell multiplication is needed. Leucocytic and other trephones probably bring to resting tissues the substances required for growth, and oppose, as is the case *in vitro*, the inhibiting influence of serum.

CONCLUSIONS.

It was not my intention in this report to give a description of all the applications of the method of tissue culture to pathological problems, but simply to show how certain technical improvements, such as the use of pure cultures of tissues, and the development of procedures permitting a quantitative study of the interactions of the tissues and their medium, have led to the discovery of some previously unknown properties of the humours and of the cells. The growth-restraining action of blood serum, the growth-promoting properties of certain substances synthesized by embryonic and other cells, and the nutritive function of the white blood cells have been revealed by the new procedures. Although its technical methods are still in their infancy and very crude, the method of tissue culture will have an important bearing upon our understanding of pathological problems because it can reduce complex phenomena to simpler terms, and bring to light some of their underlying principles.

TOOTH-PLATE REMOVED FROM THE RIGHT BRONCHUS BY INFERIOR BRONCHOSCOPY.

BY

E. B. WAGGETT, D.S.O., M.B., B.C.,

THROAT AND EAR DEPARTMENT, CHARING CROSS HOSPITAL,

AND

ERIC L. FYFFE, M.B., B.S.LOND.,

MAJOR R.A.M.C.,

SURGICAL SPECIALIST, QUEEN ALEXANDRA MILITARY HOSPITAL, MILLBANK.

(*With Special Plate.*)

THIS case is worthy of record on account of the extraordinarily large size of the foreign body; there are not many records of an unbroken dental plate recovered from a bronchus.

NOTE BY MAJOR FYFFE.

Bandsman S. was admitted to Queen Alexandra Military Hospital, Millbank, on April 26th, 1924, with the following history: On the previous day (April 25th) he was having a discussion with some men in a barrack room when one of them hit him in the mouth, thereby dislodging a tooth-plate and knocking it down his throat; he immediately had a sensation of choking, and, feeling that the tooth-plate had stuck in his throat, he put his finger into his